

FORM PTO-1390 (REV. 11-2000)		DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER 1422-0493P U.S. APPLICATION NO. (IF KNOWN, SEE 35 U.S.C. 1.5) 09/937521 NEW	
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE March 24, 2000		
PRIORITY DATE CLAIMED March 26, 1999			
TITLE OF INVENTION CERAMIDASE GENE			
APPLICANT(S) FOR DO/EO/US ITO, Makoto			
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1). 4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. WO 00/58448 c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> is transmitted herewith. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4) 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). <p>Items 11. to 20. below concern document(s) or information included:</p> <ol style="list-style-type: none"> 11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98, Form PTO-1449(s), and International Search Report (PCT/ISA/210) with 8 cited document(s). 12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information: International Preliminary Report (PCT/IPEA/409) Two (2) Sheets of Formal Drawings Sequence Listing 			

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER																					
09/937521	PCT/JP00/01802	1422-0493P																					
<p>21. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):</p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$1,000.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. \$710.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4). \$100.00</p> <p>ENTER APPROPRIATE BASIC FEE AMOUNT =</p>		CALCULATIONS	PTO USE ONLY																				
<p>Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p>		\$ 860.00																					
<table border="1"> <thead> <tr> <th>CLAIMS</th> <th>NUMBER FILED</th> <th>NUMBER EXTRA</th> <th>RATE</th> </tr> </thead> <tbody> <tr> <td>Total Claims</td> <td>25 - 20 =</td> <td>5</td> <td>X \$18.00</td> </tr> <tr> <td>Independent Claims</td> <td>2 - 3 =</td> <td>0</td> <td>X \$80.00</td> </tr> <tr> <td colspan="2">MULTIPLE DEPENDENT CLAIM(S) (if applicable)</td> <td>Yes</td> <td>+ \$270.00</td> </tr> <tr> <td colspan="4" style="text-align: center;">TOTAL OF ABOVE CALCULATIONS = \$ 1,220.00</td> </tr> </tbody> </table>		CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	Total Claims	25 - 20 =	5	X \$18.00	Independent Claims	2 - 3 =	0	X \$80.00	MULTIPLE DEPENDENT CLAIM(S) (if applicable)		Yes	+ \$270.00	TOTAL OF ABOVE CALCULATIONS = \$ 1,220.00				\$ 0.00	
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<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.		\$ 0.00																					
<p>SUBTOTAL = \$ 1,220.00</p> <p>Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).</p>		\$ 0.00																					
<p>TOTAL NATIONAL FEE = \$ 1,220.00</p> <p>Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property</p>		\$ 40.00																					
<p>TOTAL FEES ENCLOSED = \$ 1,260.00</p>																							
		Amount to be: refunded	\$																				
		charged	\$																				
<p>a. <input checked="" type="checkbox"/> A check in the amount of <u>\$ 1,260.00</u> to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>02-2448</u>.</p>																							
<p>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</p>																							
<p>Send all correspondence to: Birch, Stewart, Kolasch & Birch, LLP or Customer No. 2292 P.O. Box 747 Falls Church, VA 22040-0747 (703)205-8000</p>																							
<p>Date: <u>September 26, 2001</u> By  Marc S. Weiner, #32,181</p>																							
<p>/rem</p>																							

PATENT
1422-0493P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: ITO, Makoto Conf.:
Int'l. Appl. No.: PCT/JP00/01802
Appl. No.: New Group:
Filed: September 26, 2001 Examiner:
For: CERAMIDASE GENE

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION

Assistant Commissioner for Patents
Washington, DC 20231

September 26, 2001

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Before line 1, insert --This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/JP00/01802 which has an International filing date of March 24, 2000, which designated the United States of America and was not published in English.--

IN THE CLAIMS:

Please amend the claims as follows:

4. (Amended) A recombinant DNA comprising the gene of claim 1.

5. (Amended) An expression vector for a microorganism, an animal cell or a plant cell, comprising the gene of claim 1 or the recombinant DNA of claim 4.

9. (Amended) A polypeptide possessing a ceramidase activity, encoded by the gene of claim 1.

11. (Amended) An antisense DNA which is complementary to the gene of claim 1 or a part thereof.

12. (Amended) An Antisense RNA which is complementary to the gene of claim 1 or a part thereof.

14. (Amended) An oligonucleotide probe or primer, capable of specifically hybridizing to the gene of claim 1, or a complementary strand thereof.

15. (Amended) An antibody or a fragment thereof, capable of specifically binding to the polypeptide of claim 8.

20. (Amended) A method of controlling an amount of ceramide in a cell and/or in a tissue, characterized by introducing the gene of claim 1 or an antisense nucleic acid thereof into the cell and/or the tissue, thereby controlling the amount of ceramide in the cell and/or in the tissue.

REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application.

The claims have also been amended to delete improper multiple dependencies.

Entry of the above amendments is earnestly solicited. An early and favorable first action on the merits is earnestly solicited.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By 

Marc S. Weiner, #32,181

P.O. Box 747

Falls Church, VA 22040-0747

MSW/rem

1422-0493P

(703) 205-8000

Attachment: VERSION WITH MARKINGS TO SHOW CHANGES MADE

VERSION WITH MARKINGS TO SHOW CHANGES MADE

The specification has been amended to provide a cross-reference to the previously filed International Application.

IN THE CLAIMS:

The claims have been amended as follows:

4. (Amended) A recombinant DNA comprising the gene of [any one of claims 1 to 3] claim 1.

5. (Amended) An expression vector for a microorganism, an animal cell or a plant cell, comprising the gene of [any one of claims 1 to 3] claim 1 or the recombinant DNA of claim 4.

9. (Amended) A polypeptide possessing a ceramidase activity, encoded by the gene of [any one of claims 1 to 3] claim 1.

11. (Amended) An antisense DNA which is complementary to the gene of [any one of claims 1 to 3] claim 1 or a part thereof.

12. (Amended) An Antisense RNA which is complementary to the gene of [any one of claims 1 to 3] claim 1 or a part thereof.

14. (Amended) An oligonucleotide probe or primer, capable of specifically hybridizing to the gene of [any one of claims 1 to 3] claim 1, or a complementary stand thereof.

15. (Amended) An antibody or a fragment thereof, capable of specifically binding to the polypeptide of [any one of claims 8 to 10] claim 8.

20. (Amended) A method of controlling an amount of ceramide in a cell and/or in a tissue, characterized by introducing the gene of [any one of claims 1 to 3] claim 1 or an antisense nucleic acid thereof into the cell and/or the tissue, thereby controlling the amount of ceramide in the cell and/or in the tissue.

2/PATS

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09/937521

JC12 Rec'd PCT/EP 26 SEP 2001

DESCRIPTION

CERAMIDASE GENE

5 TECHNICAL FIELD

The present invention relates to a polypeptide possessing a ceramidase activity, an antibody specifically binding thereto, a gene encoding the polypeptide, and a probe and primer which are capable of specifically hybridizing thereto, which are useful as a reagent for lipid engineering for 10 analyzing a structure, functions, and the like of a ceramide. In addition, the present invention relates to a method for producing the above-mentioned polypeptide by a genetic engineering means, and a method for detecting the polypeptide or the gene, and a kit therefor. Further, the present invention relates to a method of controlling an amount of a ceramide in a cell and/or in a tissue, 15 which can be applied to a disease caused by abnormality in the amount of the ceramide.

BACKGROUND ART

A ceramidase is an enzyme which hydrolyzes a ceramide, a kind of a sphingolipid, into a sphingoid and a fatty acid. The sphingoid which is generated 20 by hydrolyzing the ceramide with the ceramidase possesses various physiological activities such as inhibition of protein kinase C, activation of phospholipase D and inhibition of a calmodulin-dependent enzyme. As described above, the above-mentioned sphingoid is an important substance 25 which is thought to be acting on the regulation of the cell functions because the

sphingoid is involved in proliferation of cells and intracellular signal transduction. The ceramidase is an enzyme which plays an important role of the control of the amount of the above-mentioned sphingoid.

5 Ceramidases are classified into acidic ceramidases and neutral/alkaline ceramidases by the optimum pH. There have been, so far reported that the presence of a ceramidase possessing the optimum pH in an acidic range has been found in mammalian tissues such as rat brain [*Biochemistry*, **8**, 1692-1698 (1969)], guinea pig epithelial cells [*J. Biol. Chem.*, **270**, 12677-12684 (1995)], human kidney [*Biochim. Biophys. Acta*, **398**, 125-131 (1975)], spleen [*Biochim. Biophys. Acta*, **1004**, 245-251 (1989)], fibroblasts [*Biochem. J.*, **205**, 419-425 (1982)], and epithelium [*FEBS Lett.*, **268**, 110-112 (1990)]; and human urine [*J. Biol. Chem.*, **270**, 11098-11102 (1995)], and the like.

10 In addition, it has been clarified that a bacterium belonging to the genus *Pseudomonas* produces a ceramidase, and this ceramidase is a ceramidase possessing optimum pH in an alkaline region [*J. Biol. Chem.*, **273**, 14368-14373 (1998)].

15 Among these ceramidases, amino acid sequences of the acidic ceramidase purified from human urine and nucleotide sequences of a gene encoding the ceramidase have been determined [*J. Biol. Chem.*, **271**, 33110-33115 (1996)]. In addition, an acidic ceramidase gene of a mouse has been obtained by utilizing its homology with the above-mentioned acidic ceramidase gene derived from human urine [*Genomics*, **50**, 267-274 (1998)].

20 However, since all of the ceramidase genes derived from mammals which have been reported encode acidic ceramidases, amino acid sequences and genomic structures of the neutral/alkaline ceramidase in mammals have been

completely unknown, so that biological functions of the neutral/alkaline ceramidase in higher organisms have not yet been elucidated at present.

In the studies on the elucidation of the *in vivo* functions of a ceramide, metabolic control therefor, diagnosis or treatment of a disease associated with the ceramide or the like, it is necessary to obtain detailed information concerning various enzymes associated with the ceramide, and the enzyme gene. However, as mentioned above, findings on the amino acid sequence and genes thereof of the neutral/alkaline ceramidase in mammals have not yet been obtained at present. Therefore, in order to develop the technique as described above pertaining to a ceramide, it is necessary to obtain some findings associated with a neutral/alkaline ceramidase, especially a gene thereof.

As mentioned above, although several reports have been made on cloning of ceramidase genes of a mammal, all of these reports are concerned with ceramidases possessing an activity in an acidic region, which cannot be expected to possess a homology with a ceramidase possessing an activity in a neutral/alkaline region. Therefore, it has been difficult to obtain a neutral/alkaline ceramidase gene as a homolog of a nucleotide sequence of the acidic ceramidase gene.

20 DISCLOSURE OF INVENTION

The present invention has been accomplished in view of the above-described prior art, and a first object of the present invention is to provide a neutral/alkaline ceramidase gene of a mammal. A second object of the present invention is to provide a method for producing a neutral/alkaline ceramidase gene in a high purity by a genetic engineering means, comprising using a

transformant resulting from incorporation of an expression vector carrying the gene. A third object of the present invention is to provide a polypeptide encoding the above-mentioned gene. A fourth object of the present invention is to provide an antisense DNA and an antisense RNA which are complementary to the gene of the present invention or a part thereof. A fifth object of the present invention is to provide a synthetic oligonucleotide probe or primer capable of specifically hybridizing to the gene of the present invention. A sixth object of the present invention is to provide an antibody or a fragment thereof specifically binding to the polypeptide. A seventh object of the present invention is to provide a method for detecting the above-mentioned ceramidase or a gene thereof, and a kit used therefor. An eighth object of the present invention is to provide a method of controlling an amount of ceramide in the cell or in the tissue.

The present inventors have succeeded in isolating a neutral/alkaline ceramidase from liver of a mouse, a mammalian, and isolating the genes. In addition, they have succeeded in elucidating the structure of the neutral/alkaline ceramidase of a mammal including human by using techniques such as hybridization or polymerase chain reaction (PCR). Further, they have also succeeded in conveniently producing a neutral/alkaline ceramidase in a high purity by genetic engineering techniques by using the genes. Thus, the present invention has been perfected thereby.

Specifically, the gist of the present invention relates to:

[1] a gene having a nucleotide sequence of a nucleic acid selected from the group consisting of:

(A) a nucleic acid encoding a polypeptide having the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing or a partial sequence thereof, the

- polypeptide possessing a ceramidase activity;
- (B) a nucleic acid having a nucleotide sequence of SEQ ID NO: 15 of the Sequence Listing or a partial sequence thereof and encoding a polypeptide possessing a ceramidase activity;
- 5 (C) a nucleic acid encoding a polypeptide consisting of an amino acid sequence resulting from deletion, addition, insertion or substitution of at least one amino acid residue in the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing, the polypeptide possessing a ceramidase activity;
- 10 (D) a nucleic acid consisting of a nucleotide sequence resulting from deletion, addition, insertion or substitution of at least one base in the nucleotide sequence of SEQ ID NO: 15 of the Sequence Listing and encoding a polypeptide possessing a ceramidase activity;
- (E) a nucleic acid capable of hybridizing to a complementary strand of a
- 15 nucleic acid of any one of the above (A) to (D), under stringent conditions, and encoding a polypeptide possessing a ceramidase activity; and
- (F) a nucleic acid having a nucleotide sequence different from the nucleic acid of any one of above (A) to (E) via degeneracy and encoding a polypeptide possessing a ceramidase activity;
- 20 [2] a recombinant DNA comprising the gene of item [1] above;
- [3] an expression vector for a microorganism, an animal cell or a plant cell, comprising the gene of item [1] above or the recombinant DNA of item [2] above;
- [4] a transformant carrying the expression vector of item [3] above;
- 25 [5] a method for producing a polypeptide possessing a ceramidase activity,

- characterized by culturing the transformant of item [4] above under conditions appropriate for expression of the ceramidase gene and production of the polypeptide encoded by the gene, and collecting a polypeptide possessing a ceramidase activity from the resulting culture;
- 5 [6] a polypeptide having the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing or a partial sequence thereof and possessing a ceramidase activity;
- [7] a polypeptide possessing a ceramidase activity, encoded by the gene of item [1] above;
- 10 [8] an antisense DNA which is complementary to the gene of item [1] above or a part thereof;
- [9] an antisense RNA which is complementary to the gene of item [1] above or a part thereof;
- [10] an expression vector comprising the antisense DNA of item [8] above;
- 15 [11] an oligonucleotide probe or primer, capable of specifically hybridizing to the gene of item [1] above or a complementary strand thereof;
- [12] an antibody or a fragment thereof, capable of specifically binding to the polypeptide of item [6] or item [7] above;
- [13] a method for detecting a gene encoding a polypeptide possessing a ceramidase activity, comprising using the oligonucleotide probe or primer of item [11] above;
- 20 [14] a kit for the use in detection of a gene encoding a polypeptide possessing a ceramidase activity, comprising the oligonucleotide probe and/or primer of item [11] above;
- 25 [15] a method for detecting a polypeptide possessing a ceramidase activity,

comprising using the antibody or a fragment thereof of item [12] above;
[16] a kit for the use in detection of a polypeptide possessing a ceramidase activity, comprising the antibody or a fragment thereof of item [12] above; and
[17] a method of controlling an amount of ceramide in a cell and/or in a tissue,
5 characterized by introducing the gene of item [1] above or an antisense nucleic acid thereof into the cell and/or the tissue, thereby controlling the amount of ceramide in the cell and/or in the tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figure 1 is a graph showing optimum pH of the ceramidase.

Figure 2 is a restriction endonuclease map of a DNA fragment comprising the ceramidase gene.

BEST MODE FOR CARRYING OUT THE INVENTION

15 Since information such as consensus sequences for a neutral/alkaline ceramidase has not been clarified, the present inventors have obtained the amino acid information by isolating the above-mentioned neutral/alkaline ceramidase, whereby a gene encoding the above-mentioned ceramidase could be isolated for the first time.

20 From the above findings, there could be obtained a surprising finding that the amino acid sequence of the neutral/alkaline ceramidase derived from a mouse liver of the present invention has a low homology with the amino acid sequence of a known alkaline ceramidase [previously mentioned, *J. Biol. Chem.*, 273, 14368-14373 (1998)] derived from a bacterium belonging to the genus
25 *Pseudomonas* (*Pseudomonas aeruginosa*).

The ceramidase of the present invention has been clarified for the first time as a neutral/alkaline ceramidase derived from a mammal. Therefore, the present invention is even more useful in the developments of elucidation of the *in vivo* functions of a ceramide, metabolic control therefor, and diagnosis and treatment of diseases associated with a ceramide, and the like, as compared to the known alkaline ceramidase derived from the known bacterium of the genus *Pseudomonas*.

The present invention will be described hereinbelow.

10 (1) Polypeptide Possessing Ceramidase Activity

In the present specification, the phrase "polypeptide possessing a ceramidase activity" (which may be simply referred to as "ceramidase" in the present specification) refers to an enzyme possessing an activity of hydrolyzing a ceramide to generate a sphingoid and a fatty acid as mentioned above. In addition, the term "neutral/alkaline ceramidase" refers to a ceramidase possessing an optimum pH at a pH higher than the acidic range.

As one example thereof, enzymologically chemical and physicochemical characteristics of the isolated and purified, neutral/alkaline ceramidase derived from a mouse liver in the present invention will be described.

20

1. Action

The ceramidase of the present invention acts to hydrolyze a ceramide, thereby generating a sphingoid and a fatty acid.

25 The activity of the ceramidase can be determined in accordance with the method described, for instance, in *J. Biol. Chem.*, 275, 3462-3468 (2000).

Concretely, a reaction mixture prepared by dissolving 550 pmol of C12-NBD-ceramide [*Anal. Biochem.*, **263**, 183-188 (1998)], 1.0% (W/V) sodium cholate and a suitable amount of an enzyme (ceramidase) in 20 μ l of 25 mM Tris-hydrochloric acid buffer (pH 7.5) is incubated at 37°C for 30 minutes. The reaction mixture is incubated in a boiling water bath for 5 minutes, thereby stopping the reaction. The resulting reaction mixture is further evaporated to dryness under reduced pressure. The dried solid is dissolved in chloroform/methanol = 2/1 (V/V), and developed by silica gel thin layer chromatography (developing solvent: chloroform/methanol/25% aqueous ammonia = 90/20/0.5 (V/V/V)). Thereafter, the C12-NBD-fatty acid generated by the above-mentioned reaction is quantified by using CS-9300 Chromatoscanner (manufactured by Shimadzu Corporation) at an excitation wavelength of 475 nm and a fluorescent wavelength 525 nm. One unit (U) of this enzyme (ceramidase) is defined as an amount of the enzyme required for releasing 1 micromol of the C12-NBD-fatty acid, per one minute under the above-mentioned conditions, resulting from hydrolysis of the C12-NBD-ceramide.

2. Substrate Specificity

Five milliunits of the ceramidase of the present invention is acted on 20 100 pmol of various kinds of sphingolipids, in which the fatty acid moiety is labeled with ^{14}C -radioactive isotope, in 20 ml of 25 mM Tris-hydrochloric acid buffer, pH 7.5, containing 1% sodium cholate at 37°C for 24 hours. The reaction mixture is developed on silica gel thin layer chromatography. Thereafter, the ^{14}C -labeled sphingolipids and the ^{14}C -labeled fatty acids generated by the 25 enzymatic reaction are detected and quantified by Imaging Analyzer BAS1000

(manufactured by Fuji Photo Film). The degradation ratio is calculated from the obtained values. The substrate specificities of the ceramidase of the present invention are shown in Table 1.

As shown in Table 1, the ceramidase of the present invention shows substrate specificities of: (1) hydrolyzing various N-acylsphingosines; (2) not acting on galactosylceramide, sulfatide, GM1a, or sphingomyelin; (3) acting favorably on a ceramide containing sphingenin (d18:1) than a ceramide containing sphinganine (d18:0); (4) being less likely to act a ceramide containing phytosphingosine (t18:0); and the like.

10

Table 1

Substrate (Structure)	Degradation Ratio (%)
N-Lauroylsphingosine (C12:0/d18:1)	63
N-Palmitoylsphingosine (C16:0/d18:1)	93
N-Stearoylsphingosine (C18:0/d18:1)	83
N-Palmitoylsphinganine (C16:0/d18:0)	59
N-Stearoylsphinganine (C18:0/d18:0)	40
N-Palmitoylphytosphingosine (C16:0/t18:0)	5
N-Stearoylphytosphingosine (C18:0/t18:0)	2
NBD-N-Dodecanoylsphingosine (NBD-C12:0/d18:1)	97
NBD-N-Hexanoylsphingosine (NBD-C6:0/d18:1)	2
Galactosylceramide (Galb1-1'Cer)	0
Sulfatide (HS03-3Galb1-1'Cer)	0
GM1a (Galb1-3GalNAcb1-4(NeuAca2-3)Galb1-4G1cb1-1'Cer)	0
Sphingomyelin (Choline phosphate Cer)	0

3. Optimum pH

Sixteen milliunits of the ceramidase of the present invention is acted on 5 100 pmol of C12-NBD-ceramide in 20 ml of 3,3-dimethylglutaric acid, 50 mM Tris-(hydroxymethyl)aminomethane, 50 mM 2-amino-2-methyl-1,3-propanediol at 37°C for 24 hours. The resulting reaction mixture is developed by silica gel thin layer chromatography. Next, the NBD-labeled ceramide and the NBD- labeled fatty acid generated by the enzymatic reaction are detected and quantified 10 at a detection wavelength of 525 nm by using the CS-9300 Chromatoscanner. The degradation ratio is calculated from the obtained values. Figure 1 is a graph showing the relationship between the degradation activity of the C12-NBD- labeled ceramide and pH, wherein the ordinate axis shows the degradation ratio (%) 15, and the abscissa axis shows a reaction pH. As shown in the results of Figure 1, the optimum pH of the present ceramidase is from 7.0 to 8.0.

4. Temperature Stability

The ceramidase of the present invention does not show the reduced activity when treated in 20 mM Tris-hydrochloric acid (pH 7.5) buffer containing 20 0.1% polidocanol [trade name: Lubrol PX] at 37°C for 24 hours, but shows the reduced activity by a treatment at 60°C for 1 hour, to about 30% of the activity before the treatment.

5. Molecular Weight

The molecular weight of the ceramidase of the present invention is about 25 94 kDa on SDS-PAGE (under reducing conditions). In addition, the present enzyme digested by glycopeptidase F is about 73 kDa on SDS-PAGE (under

reducing conditions).

In the present specification, one example of the “polypeptide possessing a ceramidase activity” includes a polypeptide of a naturally occurring ceramidase derived from a mouse, having the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing. Further, not only the polypeptide having the amino acid sequence, but also a polypeptide having an amino acid sequence with a mutation such as substitution, deletion, addition or insertion of one or more amino acids introduced into the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing are encompassed in the term “polypeptide possessing a ceramidase activity,” as long as the polypeptide can be found to possess a similar ceramidase activity in accordance with the activity determination by the method described above. In addition, in the above-mentioned mutation, two or more kinds of mutations may be introduced, as long as they are mutations by which the resulting polypeptide can exhibit a ceramidase activity. In the present specification, the term “amino acid sequence with a mutation introduced” encompasses any of amino acid sequences into which a mutation is artificially introduced and amino acid sequences having a naturally occurring mutation.

The ceramidase having the mutation can be concretely obtained, for instance, by selecting a polypeptide possessing a ceramidase activity for the mutated gene having a mutation in the nucleotide sequence (SEQ ID NO: 15) of the ceramidase gene described below, by the following steps:

- (a) incubating a gene expression product in a reaction mixture [composition: 550 pmol of C12-NBD-ceramide and 1.0% (W/V) sodium cholate in 20 μ l of 25 mM Tris-hydrochloric acid buffer (pH 6 to 9, preferably 6.5 to 8.5,

more preferably 7 to 8, especially preferably 7.5)] at 37°C for 30 minutes, to react the mixture; and

(b) detecting generation of a C12-NBD-fatty acid in the reaction product.

5 (2) Ceramidase Gene

The ceramidase gene in the present invention refers to a gene having a nucleotide sequence of a nucleic acid encoding the above-mentioned “polypeptide possessing a ceramidase activity,” or a nucleic acid comprising a nucleotide sequence of the gene. One example thereof includes a gene having a nucleotide sequence of a nucleic acid encoding a polypeptide consisting of the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing or a partial sequence thereof; and a gene having the nucleotide sequence of a nucleic acid consisting of a nucleotide sequence of SEQ ID NO: 15 of the Sequence Listing, or a partial sequence thereof, and the present invention is not limited to these exemplified. As described above, even a gene having a nucleotide sequence of a nucleic acid encoding a polypeptide consisting of a partial sequence of the amino acid sequence of SEQ ID NO: 14, or a gene having a nucleotide sequence of a nucleic acid consisting of a partial sequence of the nucleotide sequence of SEQ ID NO: 15 are encompassed in the scope of the present invention, as long as the gene encodes a polypeptide possessing a ceramidase activity. These genes are neutral/alkaline ceramidase genes derived from mouse liver, and the origin of the ceramidase gene of the present invention is not particularly limited, as long as the ceramidase activity of the gene product can be detected in the same manner as in the steps (a) and (b) described in item (1) above. The origin includes, for instance, mice, rats, humans, hamsters, guinea pigs, and the like.

In the present specification, the phrase "polypeptide consisting of a partial sequence (of the amino acid sequence)" means those in which the ceramidase activity can be detected by the steps (a) and (b) described in item (1) above.

Further, a gene encoding a ceramidase having a mutation, which can
5 exhibit a similar ceramidase activity, is also encompassed in the present
invention. For instance, even a gene having a nucleotide sequence of a nucleic
acid encoding an amino acid sequence with a mutation such as deletion, addition,
insertion or substitution of one or more amino acid residues introduced into the
amino acid sequence of SEQ ID NO: 15 of the Sequence Listing is encompassed
10 in the gene of the present invention, as long as the polypeptide encoded by the
gene possesses a ceramidase activity. Even a naturally occurring gene having a
mutation as well as an artificially prepared gene described above are
encompassed in the scope of the present invention, as long as the gene is a gene
15 having a nucleotide sequence of a nucleic acid encoding a polypeptide
possessing an activity for a neutral/alkaline ceramidase.

As the method for preparing a gene into which a mutation is artificially introduced as described above, for instance, the following method is used.

As a method of introducing a random mutation, there can be employed, for instance, a method for generating a transition mutation in which cytosine base is substituted by uracil base with a chemical treatment using sodium hydrogen sulfite [*Proceedings of the National Academy of Sciences of the USA*, 79, 1408-1412 (1982)]; a method for lowering an accuracy of incorporation of nucleotide during DNA synthesis by carrying out PCR in a reaction mixture containing manganese [*Anal. Biochem.*, 224, 347-353 (1995)]; and the like.

25 As a method for introducing site-directed mutagenesis, there can be

employed, for instance, a method utilizing amber mutation [gapped duplex method, *Nucleic Acids Research*, 12, 9441-9456 (1984)]; a method of utilizing a *dut* (dUTPase) gene and *ung* (uracil-DNA glycosilase) gene deficient host [Kunkel method, *Proceedings of the National Academy of Sciences of the USA*, 82, 488-492 (1985)]; a method by PCR utilizing amber mutation (WO 98/02535); and the like. Various kinds of kits for introducing site-directed mutagenesis to a desired gene by these methods are commercially available, and a gene resulting from introduction of a desired mutation can be readily obtained by utilizing the kits.

10 In addition, the nucleic acid capable of hybridizing to a complementary strand of the above-mentioned nucleic acid under stringent conditions, the gene having a nucleotide sequence of a nucleic acid encoding a polypeptide possessing a ceramidase activity is also encompassed in the gene of the present invention. The ceramidase activity can be detected, for instance, by the steps (a) and (b) described in item (1) above.

15 Here, the term "stringent conditions" refers to, for instance, the following conditions. Concretely, the term refers to conditions of carrying out incubation at 50°C for 4 hours to overnight in 6 x SSC (wherein 1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.5% SDS, 5 x Denhardt's [0.1% bovine serum albumin (BSA), 0.1% polyvinyl pyrrolidone, 0.1% Ficol 400], and 20 100 µg/ml salmon sperm DNA.

Also, the details of the hybridization manipulations are described, for instance, in *Molecular Cloning: A Laboratory Manual*, 2nd Ed., published by Cold Spring Harbor Laboratory in 1989, edited by T. Maniatis et al.

25 The gene having a nucleotide sequence different from the nucleic acid

mentioned above via a degenerated genetic code is also encompassed in the ceramidase gene of the present invention.

The polypeptide encoded by the gene of the present invention is encompassed in the present invention, as long as its ceramidase activity can be detected by the steps (a) and (b) described in item (1) above.

According to the gene of the present invention, there is further provided a recombinant DNA suitable for various purposes used. Here, the term “recombinant DNA” refers to a DNA carrying the gene of the present invention, obtained by genetic engineering technique.

10 The recombinant DNA carrying the ceramidase gene of the present invention is ligated to a known vector or the like, whereby an expression vector inserted with the ceramidase gene in an expressible state can be prepared. Such an expression vector is also encompassed in the present invention.

In the present invention, an expression vector refers to a vector which is constructed such that the gene or recombinant DNA mentioned above is inserted and expressed in desired host cells. In addition, a vector in which an antisense DNA as described below is inserted is also encompassed in the expression vector of the present invention. The vector to be inserted includes plasmid vectors, phage vectors, viral vectors, and the like. As the plasmid vector, commercially available products such as pUC18, pUC19, pBluescript, and pET can be suitably used, and as the phage vectors, commercially available products of lambda phage vectors such as λgt10 and λgt11 can be suitably used, without being limited thereto. As the viral vectors, retroviral vector, adenoviral vector, vaccinia viral vector, adeno-associated viral vector, and the like can be used, without being limited thereto. These vectors are appropriately selected in accordance with the

host cells used. Each of these vectors may appropriately carry a factor such as an inducible promoter, a selectable marker gene, or a terminator.

In addition, in order to facilitate isolation and purification, a vector carrying a sequence capable of expressing as His tag or GST fusion protein may be used depending upon its use. In this case, GST (glutathione S-transferase) fusion protein vector carrying an appropriate promoter (for instance, *lac*, *tac*, *trc*, *trp*, CMV, SV40 early promoter, or the like) which functions in a host cell (for instance, pGEX4T), a vector carrying a tag (for instance, Myc, HisA, or the like) sequence or the like can be used.

10

(3) Transformant Harboring Ceramidase Gene

The transformant of the present invention, namely the cells capable of expressing the ceramidase gene of the present invention, can be obtained by transforming a host with an expression vector in which the ceramidase gene of the present invention is inserted. The host used can be appropriately selected depending upon the purposes used of the desired ceramidase, and microorganisms such as *Escherichia coli*, yeasts, animal cells, plant cells, animal individuals, plant individuals, and the like. Concretely, *Escherichia coli* includes HB101 strain, C600 strain, JM109 strain, DH5 α strain, DH10B strain, XL-1BlueMRF' strain, TOP10F strain and the like of the *Escherichia coli* K-12 derivative. Also, the yeast cells include *Saccharomyces cerevisiae* and the like. The animal cells include L, 3T3, FM3A, CHO, COS, Vero, Hela, and the like. The plant cells include tobacco BY2 and the like.

As a method for transducing an expression vector into a host, a method, including, for instance, described in *Molecular Cloning: A Laboratory Manual*,

2nd Ed., 249-254 may be employed. Next, in order to select a transformant expressing a desired gene, the characteristics of the expression vector are utilized. For instance, in a case where a plasmid vector is pBluescript and a host cell *Escherichia coli*, a colony having ampicillin resistance on a plate containing ampicillin is selected, or a colony having ampicillin resistance and showing white color on a plate containing ampicillin, 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) and isopropyl- β -D-thiogalactopyranoside (IPTG) is selected, thereby selecting a colony in which a foreign gene is transduced.

The polypeptide possessing a ceramidase activity can be produced by culturing the transformant of the present invention under generally employed conditions. In some cases, the codon usage differs depending upon a host in which the gene of the present invention is expressed, so that expression is suppressed. In this case, the codon used in the gene of the present invention may be changed to a codon suitable to each host. In addition, the above-mentioned expression vector is not limited only to those vectors derived from plasmids, and vectors derived from phages, cosmids, and the like may also be used, as long as the object of the present invention is not hindered. A vector capable of inducing and expressing a foreign gene, a vector capable of expressing as a fusion protein with a reporter gene product, and the like are desirable, from the viewpoint of readily and massively producing the polypeptide of the present invention.

The expression of the ceramidase can be confirmed by determining the ceramidase activity. The activity can be determined by, for instance, the method described in *J. Biol. Chem.*, 275, 3462-3468 (2000) using a cell extract of the transformant as a sample, for instance, similar procedures to the steps (a) and (b) described in item (1) above. In addition, the expression of the ceramidase can be

confirmed by determining the amount of a ceramide in the cell. The above-mentioned amount of ceramide can be determined, for instance, by the method described in *Analytical Biochemistry*, 244, 291-300 (1997). Also, an antibody against the ceramidase can be used. In a case where the ceramidase is expressed as a fusion body with another polypeptide (the polypeptide excluding the ceramidase of the present invention), an antibody against this polypeptide moiety, the polypeptide excluding the ceramidase of the present invention, may be used. In a case where an antibody is used, the ceramidase can be detected by, for instance, subjecting a cell extract of the transformant to electrophoresis on an SDS-polyacrylamide gel, and thereafter transferring the electrophoresed gel on a polyvinylidene fluoride (PVDF) membrane, and detecting with the antibody on this membrane.

(4) Method for Producing Polypeptide Possessing Ceramidase Activity

The present invention also provides a method for producing a polypeptide possessing a ceramidase activity, comprising the steps of culturing the above-mentioned transformant under conditions appropriate for expression of the ceramidase gene of the present invention and production of the polypeptide encoding the gene, and collecting a polypeptide possessing a ceramidase activity from the resulting culture. The method for culturing the transformant is not particularly limited, and an appropriate one can be selected from a known culturing method appropriate for the host used.

In the production method of the present invention, in a case where the above-mentioned transformant is a microorganism or a cultured cell, the ceramidase can be efficiently produced by determining optimum conditions for

expression of the ceramidase in a medium composition, a pH of a medium, a culturing temperature, and a culturing time, as well as the amount of an inducer used, and the using time period, and the like.

A general method is employed in the purification of the ceramidase from the culture of the transformant. In a case where the transformant intracellularly accumulates the ceramidase as in the case of *Escherichia coli*, after the termination of culture, the transformant cells are harvested by centrifugation, and the resulting cells are disrupted by sonication or the like, and thereafter centrifuged or the like to give a cell-free extract. The cell-free extract used as a starting material can be purified by salting-out as well as a general protein purification method such as various kinds of chromatographies such as ion-exchange chromatography, gel filtration chromatography, hydrophobic chromatography, and affinity chromatography. The expression product may be extracellularly secreted depending upon the host-vector system used in some cases. In such a case, the purification can be similarly carried out from culture supernatant.

According to the method of the present invention, in a case where the ceramidase is produced intracellularly, a desired ceramidase may coexist intracellularly with impurities such as various kinds of enzymes and proteins. Since the impurities are present in a trace amount as compared to the amount of the expressed ceramidase, there is an excellent advantage that its purification is extremely facilitated. In addition, in a case where an extracellular secretion-type vector is used as a vector, the ceramidase is extracellularly secreted, so that the medium component or the like coexists in the fraction comprising the ceramidase. However, since the fraction usually contains almost no protein component which

would hinder the ceramidase purification, there is an excellent advantage in that, for instance, complicated separation and purification procedures which have been required in the purification of the ceramidase from a mouse liver are not necessitated.

5 In addition, in a case of a ceramidase derived from a fungus, there is a possibility that the enzyme itself has a sugar chain. A polypeptide possessing a ceramidase activity and having no sugar chain can be produced by using as a host cell a cell which does not possess a sugar chain-synthesizing ability, for instance, a mutant cell which has lost a sugar chain-synthesizing ability of a 10 prokaryote such as *Escherichia coli*, *Bacillus substillis* or *Actinomyces*, or an yeast, a fungus, an animal cell, an insect cell and a plant cell. Further, an enzyme having a sugar chain can be produced. In this case, a polypeptide possessing a ceramidase activity and having a sugar chain can be produced by using as a host cell a cell which possesses a sugar chain-synthesizing ability, for 15 instance, an yeast, a fungus, an animal cell, an insect cell and a plant cell.

Also, the expression product may form an insoluble inclusion body depending upon the host-vector system used. In this case, after the termination of culture, the transformant cells are harvested by centrifugation, and the resulting cells are disrupted by sonication or the like, and thereafter centrifuged 20 or the like, thereby harvesting an insoluble fraction containing the inclusion body. After washing the inclusion body, the inclusion body is solubilized with an generally used protein solubilizing agent, for instance, urea, guanidine hydrochloride, or the like, and purified by various kinds of chromatographies such as ion-exchange chromatography, gel filtration chromatography, 25 hydrophobic chromatography, and affinity chromatography as occasion demands.

Thereafter, refolding procedures employing dialysis or dilution method are carried out, whereby a preparation comprising a polypeptide possessing a ceramidase activity can be obtained. If this preparation is further purified by various kinds of chromatographies as occasion demands, a polypeptide 5 possessing a ceramidase activity in a high purity can be obtained.

(5) Probe for Hybridization and Primer for PCR

The oligonucleotide probe or primer of the present invention is capable of specifically hybridizing to the gene of the present invention, or a complementary 10 strand thereof. The oligonucleotide probe or primer is designed on the basis of the nucleotide sequence of the ceramidase gene of the present invention. For instance, the oligonucleotide probe or primer can be prepared by chemical 15 synthesis by a general method. The nucleotide sequence for the oligonucleotide probe is not particularly limited. The nucleotide sequence is those capable of hybridizing to the above-mentioned ceramidase gene, or a nucleic acid having a nucleotide sequence complementary to the gene under stringent conditions. The 20 above-mentioned term "stringent conditions" is not particularly limited. For instance, the term "stringent conditions" refers to conditions of incubating overnight at a temperature of $[(T_m \text{ of the above-mentioned probe}) - 25^\circ\text{C}]$ in a 25 solution containing 6 x SSC, 0.5% SDS, 5 x Denhardt's, and 100 mg/ml salmon sperm DNA, and the like. Also, the nucleotide sequence of the above-mentioned primer is not particularly limited, as long as the primer is annealed to the above-mentioned ceramidase gene or the gene having a nucleotide sequence complementary to the gene under usual reaction conditions for PCR so that the extension reaction by the DNA polymerase can be initiated.

Tm of the oligonucleotide probe or primer can be calculated, for instance, by the following equation:

$$Tm = 81.5 - 16.6 (\log_{10}[\text{Na}^+]) + 0.41 (\%G + C) - (600/N)$$

wherein N is a chain length of the oligonucleotide probe or primer; and

5 %G + C is a content of guanine and cytosine residues in the oligonucleotide probe or primer.

In addition, when the chain length of the oligonucleotide probe or primer is shorter than 18 bases, *Tm* can be deduced from a product of the contents of 10 A + T (adenine + thymine) residues multiplied by 2°C, with a sum of a product of the contents of G + C residues multiplied by 4°C [(A + T) x 2 + (G + C) x 4].

The chain length of the above-mentioned oligonucleotide probe is not particularly limited. It is preferable that the chain length is 15 bases or more, more preferably 18 bases or more, from the viewpoint of preventing nonspecific 15 hybridization.

In addition, as the primer of the present invention, there can be cited the nucleic acids having the same nucleotide sequences as those for the above-mentioned oligonucleotide probe. For instance, the primer can be prepared by, for instance, designing on the basis of the nucleotide sequence of the gene of the 20 present invention, and chemically synthesizing it, and the like. The chain length of the primer is not particularly limited. For instance, the primer having a chain length of 15 to 40 bases can be used, especially one having a chain length of 17 to 30 bases can be suitably used. The above-mentioned primer can be used for various gene amplification methods such as PCR method, whereby the 25 ceramidase gene of the present invention can be detected.

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Also, as the above-mentioned oligonucleotide probe or primer, there may be used a nucleic acid obtained by fragmenting a nucleic acid encoding a naturally occurring ceramidase by an enzymatic treatment such as endonuclease treatment or exonuclease treatment, a physical treatment such as sonication, or the like, and subjecting the resulting fragment to separation and purification by various kinds of nucleic acid separation methods represented by agarose gel or the like. It is desired that the nucleic acid obtained as described above is derived from a region having a sequence characteristic of the ceramidase.

Further, in order to more readily detect the nucleic acid to be detected, the above-mentioned oligonucleotide probe or primer can be subjected to appropriate labeling in accordance with a known method to be used in the detection of the ceramidase gene of the present invention. The labeling is not particularly limited. The oligonucleotide probe or primer may be labeled with radioisotopes as well as various labels represented by fluorescent substances, and ligands such as biotin and digoxigenin.

A DNA having high homology to the ceramidase gene of the present invention can be cloned by screening a genomic DNA or cDNA derived from an organ other than a mouse liver or from an organism other than a mouse, or a genomic DNA library or cDNA library, by using the probe for hybridization of the present invention.

In addition, a DNA fragment having high homology to the ceramidase gene of the present invention can be detected by PCR method from a genomic DNA or cDNA derived from an organ other than mouse liver or derived from an organism other than mouse, or a genomic DNA library or cDNA library, by using the primer of the present invention, and further its full length gene can also

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be obtained.

(6) Method for Detecting Gene

One of the big features of the method for detecting a gene of the present invention resides in that the gene in the sample to be detected is detected by using the above-mentioned oligonucleotide probe and/or primer.

In the method for detection of the present invention, the gene may be detected by hybridization method or the like by using the above-mentioned oligonucleotide probe, or the gene may be detected by DNA amplification method such as PCR method by using the above-mentioned primer.

In a case of hybridization using the oligonucleotide probe, the samples to be detected include, for instance, samples such as colonies and cultured cells of microorganisms, and tissue fragments, those obtained by immobilizing DNA or RNA in these samples onto a membrane, DNA or RNA extracted from these samples, and the like.

The hybridization can be carried out in accordance with a known method described in *Molecular Cloning: A Laboratory Manual*, 2nd Ed. and the like. The conditions for the hybridization can be appropriately determined by the T_m value of the probe used, the GC content of the target DNA, and the like. For instance, the conditions described in *Molecular Cloning: A Laboratory Manual*, 2nd Ed. mentioned above or the like can be applied.

In a case where the gene is detected by using the primer, the samples to be detected include, for instance, microorganism samples such as culture of microorganisms, colonies of microorganisms and bacterial cells of microorganisms; samples derived from a living body such as cultured cells,

tissues and tissue fragments; and the like. As these samples, for instance, isolated microorganisms and cultured cells may be used in the original states, or after being subjected to an appropriate treatment. In addition, solid samples such as tissues can be used by preparing an exudate or suspension. Also, supernatant 5 of these samples, or those samples prepared by subjecting these samples to a cytolytic treatment such as a treatment with a surfactant, or supernatant thereof can be used. Further, the sample may be subjected to a procedure of removing other components in the sample within a range so as not to impair the nucleic acid to be detected.

10 In a case where the detection is carried out by PCR method by using the above-mentioned primer, the PCR conditions can be appropriately selected in accordance with the *Tm* value of the primer used, the length of the region to be amplified and detected, and the like. In PCR, the desired gene can be detected by confirming the presence or absence of the amplified product. The method for 15 confirming the presence or absence of amplification is not particularly limited. For instance, the confirmation can be made by, for instance, subjecting a reaction mixture for nucleic acid amplification to agarose gel electrophoresis, staining the gel with an appropriate nucleic acid staining reagent, such as ethidium bromide or SYBER Green I, subjecting the gel to irradiation with ultraviolet rays, and 20 detecting the presence or absence of the band. The detection of the band may be observed by naked eyes, or the detection can be made, for instance, by using a fluorescent image analyzer or the like.

25 In the method for detecting the gene of the present invention, in order to increase the detection sensitivity, the above-mentioned probe and primer may be used together. For instance, the gene can be detected in high sensitivity and

accurately by amplifying the ceramidase gene existing in a trace amount in the sample by PCR method with the above-mentioned primer, and thereafter hybridizing the gene with the probe.

In a case where the ceramidase gene is detected by the method for
5 detection of the present invention and the amount of the gene is further
determined, the amount of the gene can be determined by quantifying an
intensity of the signal ascribed to the hybridized probe, a fluorescent intensity of
the band ascribed to a product amplified with the primer, or the like. The
expression amount of the desired gene can be examined by quantifying the
10 amount by using mRNA as an object to be determined.

In addition, in the method for detection of the present invention, the
detection can be carried out more conveniently by using the kit for the use in
detection of the gene of the present invention. Such a kit is also encompassed in
the present invention. One of the features of the above-mentioned kit resides in
15 that the kit comprises the above-mentioned oligonucleotide probe and/or the
above-mentioned primer. The above-mentioned kit may contain various
components used in the detection procedures. For instance, in a case of a kit
comprising an oligonucleotide probe, there may be contained various kinds of
reagents for hybridization representatively exemplified by a membrane for
20 immobilizing a nucleic acid, a hybridization buffer, and the like. Also, in a case
of a kit comprising a primer, there may be contained reagents for PCR
representatively exemplified by thermostable DNA polymerases, dNTP mixed
solutions, buffers for PCR, and the like. Further, there may be contained
reagents for detecting a probe or an amplified DNA, media for proliferating
25 microorganisms, media for culturing cells, reagents for extracting nucleic acids

from a sample, and the like.

(7) Antibody or Fragment Thereof Specifically Binding to Polypeptide Possessing Ceramidase Activity

5 The antibody or a fragment thereof specifically binding to the polypeptide of the present invention is not particularly limited, as long as the antibody or a fragment thereof possesses an ability of specifically binding to the polypeptide. The antibody may be any of polyclonal antibodies and monoclonal antibodies. Further, antibodies modified by known techniques or antibody derivatives, for 10 instance, humanized antibodies, Fab fragments, single-chain antibodies, and the like, can also be used. The antibody of the present invention can be readily prepared by appropriately immunizing a rabbit, a rat or a mouse using all or a part of the polypeptide of the present invention in accordance with the method described in, for instance, *Current Protocols in Immunology*, edited by John E. 15 Coligan, published by John Wiley & Sons, Inc., 1992. The antibody thus obtained is purified and thereafter treated with a peptidase or the like, to give an antibody fragment. In addition, the antibody can be prepared by genetically engineering means. Further, the antibody or a fragment thereof of the present invention may be subjected to various modifications in order to facilitate the 20 detection by enzyme immunoassay, fluoroimmunoassay, luminescent immunoassay, or the like.

The antibody or a fragment thereof mentioned above encompasses those which are capable of specifically binding to a certain partial fragment of the polypeptide.

25 The use of the resulting antibody or a fragment thereof includes

applications to detection of ceramidase-producing bacteria, detection of ceramidase-expressed cell lines, detection of ceramidase proteins in the cultured cell or in the tissue, affinity chromatography, screening of an expression product of various kinds of libraries (genomic DNA or cDNA), pharmaceuticals, 5 diagnostic agents, reagents for researches, and the like.

(8) Method for Detecting Polypeptide

A feature of the method for detecting the polypeptide of the present invention resides in that the polypeptide possessing a ceramidase activity is 10 detected by the antibody or a fragment thereof mentioned above.

In the present invention, as the samples to be detected, there can be used, for instance, cultures of microorganisms and animal cells, tissue fragments, cell disruptions of microorganisms and animal cells, extracts or washings of tissues such as skins, and protein samples such as membranes immobilized with proteins 15 derived from microorganisms, animal cells and tissues.

As to the detection of the specific binding of the antibody or a fragment thereof to the above-mentioned polypeptide, a known method can be utilized, and such a method includes, for instance, enzyme immunoassay, fluoroimmunoassay, luminescent immunoassay, and the like.

20 In the method for detecting the polypeptide of the present invention, the detection can be more conveniently carried out by using the kit for the use in the detection of the polypeptide of the present invention. Such a kit is also encompassed in the present invention. A feature of the above-mentioned kit resides in that the kit comprises the above-mentioned antibody or a fragment thereof. In addition, the kit may contain a reaction buffer, a labeled secondary 25

antibody, a developing reagent, and the like.

(9) Antisense DNA and Antisense RNA

In the present invention, each of the terms "antisense DNA" and "antisense RNA" refers to those having a nucleotide sequence complementary to the ceramidase gene of the present invention or a part thereof, which suppresses or controls expression (transcription, translation) of the genetic information from the gene by forming a double strand with an endogenous ceramidase gene (genomic DNA and mRNA). The length of the antisense DNA or antisense RNA can be changed depending upon the specificity of the nucleotide sequence and the method for transducing the nucleotide sequence into a cell. The antisense DNA or antisense RNA can be prepared by artificially synthesizing with a synthesizer; expressing a gene in an opposite direction (direction of antisense) of the usual direction by an enzymatic reaction with the gene of the present invention as a template; or the like. In a case where expression of the antisense RNA is desired in a living body, an expression vector ligated to the gene of the present invention in an opposite direction of the usual direction is constructed, and the resulting expression vector may be transduced into a living body.

For instance, numerous antisense techniques such as suppression of proliferation of HIV utilizing *tat* gene [*Nucleic Acids Research*, **19**, 3359-3368 (1991)] or *rev* gene [*Proceedings of the National Academy of Sciences of the USA*, **86**, 4244-4248 (1989)] have been known. Therefore, according to these methods, expression of the endogenous ceramidase gene can be suppressed or controlled by using the antisense DNA or antisense RNA of the present invention.

In addition, the antisense DNA or antisense RNA of the present invention can be utilized as a research reagent for *in situ* hybridization or the like.

5 (10) Control of Amount of Ceramide in Cell or in Tissue Using Ceramidase Gene or Antisense Nucleic Acid Thereof

10 By the gene of the present invention, a method of controlling an amount of a ceramide in a cell and/or in a tissue can be further provided. Such a method of control is also encompassed in the present invention. One of the features of the method of controlling an amount of a ceramide in a cell and/or in a tissue of 10 the present invention resides in that the ceramidase gene of the present invention is introduced into the cell and/or into the tissue, thereby controlling the amount of a ceramide in the cell and/or in the tissue.

15 Concretely, in the method of control of the present invention, the ceramidase gene of the present invention is introduced into the cell and/or into the tissue, and the ceramide is degraded in a cell or in a tissue by an action of the ceramidase expressed by the gene. On the other hand, the ceramidase gene of the present invention is introduced into the cell or into the tissue so as to generate the antisense nucleic acid of the gene, for instance, an antisense RNA, whereby the ceramidase activity in the cell or in the tissue is lowered, so that the 20 degradation of the ceramide can be suppressed. In addition, in a case where the amount of the ceramide is suppressed, the antisense nucleic acid of the ceramidase gene of the present invention, namely the antisense DNA or antisense RNA of the present invention, may be introduced in a cell or in a tissue in an intact form.

25 As the method for introducing the ceramidase gene or an antisense nucleic

acid thereof mentioned above in a cell or in a tissue, a known method can be used, and methods for physically introducing a gene such as electroporation method and particle gun method, or a method for introducing a gene using a viral vector can be utilized. The viral vector which can be used in the method of the present invention is not particularly limited. For instance, the gene can be introduced by using retroviral vector, adenoviral vector, vaccinia viral vector, adeno-associated viral vector, or the like.

According to the method of control of the present invention, the ceramidase gene or an antisense nucleic acid thereof of the present invention is introduced in a cell or in a tissue to control the amount of a ceramide in the cell and/or in the tissue, whereby exhibiting excellent effects such that a disease caused by an abnormal amount of a ceramide can be treated, and that a model animal suffering from a disease with abnormal ceramide metabolism can be prepared. The "disease caused by an abnormal amount of a ceramide" is not particularly limited, and includes, for instance, Farber's disease and the like.

The method for obtaining a ceramidase gene derived from a mouse liver will be explained hereinbelow.

1) First, a membrane fraction is prepared from a homogenate of a mouse liver, and suspended in a sucrose-EDTA solution, and the suspension is frozen and thawed, and thereafter centrifuged, to give supernatant (crude enzyme extract). A ceramidase preparation homogeneously purified can be obtained from the resulting crude enzyme extract by combining known protein purification methods, for instance, various kinds of chromatographies. As the chromatographies which can be used in the above-mentioned purification, anion

exchange chromatography, hydrophobic chromatography, chelating chromatography, gel filtration chromatography, and the like can be used.

2) Next, as the information for preparing a probe for cloning the ceramidase gene, the partial amino acid sequences of the ceramidase are examined. The

5 N-terminal amino acid sequence of the ceramidase can be found by subjecting

the above-mentioned purification ceramidase preparation itself to amino acid sequencing by Edman degradation method [*J. Biol. Chem.*, **256**, 7990-7997

(1981)]. In addition, the partial amino acid sequence of the internal ceramidase

can be obtained by purifying an appropriate peptide fragment from a peptide

10 mixture resulting from digestion of the purified enzyme preparation with a protease having a high substrate specificity, for instance, lysylendopeptidase,

N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-trypsin, and the like, and

subjecting the resulting fragment to amino acid sequencing.

3) On the basis of the information of the amino acid sequence thus clarified,

15 an oligonucleotide to be used for the probe for hybridization, or used for the primer for PCR is designed, to clone the ceramidase gene of the present

invention. In order to carry the cloning, PCR or hybridization method generally employed is utilized. PCR method can be carried out in accordance with the

method described in *PCR Technology*, edited by Erlich, H.A., published by

20 Stockton Press, 1989. The hybridization method can be carried out, for instance,

in accordance with a known method described in *Molecular Cloning: A*

Laboratory Manual, 2nd Ed.

4) As to the DNA fragment obtained by the hybridization or PCR mentioned

above, by decoding its nucleotide sequence, the amino acid sequence which can

25 be encoded therein can be known. Whether or not the above-mentioned DNA

fragment is a fragment of the ceramidase gene can be confirmed by comparing the sequence with the partial amino acid sequence of the ceramidase obtained in item 2) above.

- 5) In a case where the DNA fragment obtained by hybridization or PCR in item 3) is a part of the ceramidase gene, a DNA fragment comprising a gene encoding a full length ceramidase can be obtained by repeating the procedures of item 3), or alternatively, preparing a new probe or primer on the basis of the nucleotide sequence of the DNA fragment obtained in item 3), and carrying out hybridization or PCR by using the probe or primer.,
- 10 6) An expression vector is constructed by ligating the gene encoding a full length ceramidase thus obtained with an appropriate vector, and then the transformant in which the expression vector is transduced is prepared. The transformant is cultured, and the ceramidase activity in the resulting culture is examined, whereby confirmation can be made such that the resulting gene is one encoding the ceramidase.
- 15

However, in the cloning of the ceramidase gene of the present invention, a probe DNA appropriate for screening of the library by hybridization method could not be designed on the basis of the partial amino acid sequence information obtained in the present invention. In addition, although various PCR primers were designed by designing from each of the partial amino acid sequences and the nucleotide sequences of vector for the use in the library preparation, and the resulting primers were used in various combination to carry out PCR, specific amplification was not found, the only amplification being found alone in the combination of two kinds of primers designed on the basis of the partial amino acid sequence C-53 (shown in the amino acid sequence of C-53

20
25

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in SEQ ID NO: 3 of Sequence Listing). However, the amplified DNA fragment P-1 (shown in the nucleotide sequence of P-1 in SEQ ID NO: 6 of Sequence Listing) was as short as 68 bp, so that the amplified fragment itself could not be used as a probe for library screening by hybridization method. Therefore, a gene fragment of a size of 335 bp, which is thought to be appropriate for the first time as a probe for library screening by hybridization method, has been successfully obtained for the first time by further designing a primer for PCR on the basis of the sequence of P-1, and at the same time carrying out PCR in combination of the primer and a primer designed on the basis of the nucleotide sequence of a vector used in the preparation of the library.

Further, a gene encoding a full length ceramidase can be cloned by screening cDNA library derived from a mouse liver with the above-mentioned fragment of a size of 335 bp as a probe. Also, the genomic DNA of a ceramidase of the present invention can be obtained by screening a genomic DNA library derived from a mouse liver.

The entire nucleotide sequence of the produced ceramidase gene from a mouse liver thus obtained is shown in SEQ ID NO: 12 of Sequence Listing, and the amino acid sequence of the polypeptide encoded thereby is shown in SEQ ID NO: 13 of Sequence Listing. It has been found that the enzyme is processed to a mature enzyme in which the N-terminal part of the peptide of the gene is removed *in vivo* on the bases of this amino acid sequence and the N-terminal amino acid sequence of the gene. The amino acid sequence of this mature ceramidase and the nucleotide sequence encoded by the sequence are shown in SEQ ID NOs: 14 and 15 of Sequence Listing, respectively. Each of the above-mentioned amino acid sequence and nucleotide sequence does not have

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homology with each of the known amino acid sequence and nucleotide sequence of a ceramidase derived from a mammal. In other words, the ceramidase gene provided by the present invention consists of a completely novel sequence, which is irrelevant to the known ceramidase gene.

5 As described above, according to the present invention, there are provided a primary structure and a genetic structure of the ceramidase derived from a mouse liver. Further, there can be carried out a method for producing a polypeptide possessing a ceramidase activity inexpensively and in a high purity by genetic engineering means.

10 In addition, the oligonucleotide probe or primer capable of specifically hybridizing to the ceramidase gene of the present invention is useful in searching, detection, amplification and the like of the ceramidase gene of the present invention. The antibody or a fragment thereof specifically binding to the polypeptide of the present invention is useful in detection, identification, 15 purification, and the like of a ceramidase.

15 In addition, according to the method of controlling an amount of a ceramide in a cell and/or in a tissue of the present invention, the amount of a ceramide in the cell and/or in the tissue can be controlled by introducing the ceramidase gene of the present invention or its antisense nucleic acid into the cell 20 and/or into the tissue. Therefore, such a method of control is useful in the treatment of a disease caused by an abnormal ceramide amount, for instance, but not being particularly limited thereto, a treatment of a disease such as Farber's disease.

25 The present invention will be concretely explained by the examples,

without by no means intending to limit the scope of the present invention to these examples.

Example 1 Purification of Ceramidase

5 In 300 ml of a 0.25 M sucrose solution containing 1 mM EDTA (sucrose-EDTA solution) was homogenized 181 g of livers excised from 105 Sea/ddY mice (produced by Seiwa Experimental Animals). The resulting homogenate was centrifuged at 600 x g for 10 minutes, and thereafter the supernatant was collected. The resulting supernatant was further centrifuged at 2700 x g for 10 minutes, and the precipitates were collected.

10 The precipitated fraction was suspended in 480 ml of the sucrose-EDTA solution to give a suspension. The resulting suspension was frozen at -80°C, and thereafter thawed under running water. This freezing-thawing treatment was repeated twice. Thereafter, the treated suspension was centrifuged at 105000 x g for 90 minutes, and supernatant and precipitates were each collected. The precipitates were subjected to the treatments of freezing-thawing and centrifugation in the same manner as described above. The supernatant was collected, and combined with the previously obtained supernatant, to give 520 ml of a crude enzyme extract.

15 Two-hundred and sixty milliliters of the crude extract was applied onto 100-ml DEAE-Sepharose FF (manufactured by Amersham Pharmacia) column equilibrated with 20 mM phosphate buffer (pH 7.0) and then non-adsorbent substances were removed by wash. Thereafter, the elution was carried out with the same buffer containing 1 M NaCl to collect 160 ml of an active fraction for 20 ceramidase. The fraction was subsequently applied onto 100-ml Phenyl-

Sepharose FF (manufactured by Amersham Pharmacia) column equilibrated with 20 mM Tris-hydrochloric acid buffer (pH 7.5) containing 1 M NaCl. Thereafter, the elution was carried out on a concentration gradient of 2 to 0 M NaCl, and the elution was further carried out on a concentration gradient of 0 to 1%

5 Polidocanol (trade name: Lubrol PX, manufactured by Nacalai Tesque Inc.). By this chromatography, 310 ml of an active fraction for ceramidase was collected.

The resulting active fraction was applied onto 25-ml Chelating-Sepharose FF (manufactured by Amersham Pharmacia, Cu²⁺ bound type) column

10 equilibrated with 20 mM Tris-hydrochloric acid buffer (pH 7.5) containing

0.5 M NaCl and 0.1% Lubrol PX. The column was washed with the same buffer and then with 20 mM Tris-hydrochloric acid buffer (pH 7.5) containing 0.1%

15 Lubrol PX. Thereafter, the elution of the enzyme was carried out with 20 mM Tris-hydrochloric acid buffer (pH 7.5) containing 2 M NH₄Cl and 0.1% Lubrol

PX. The eluted active fraction was concentrated by ultrafiltration, to give a concentrate. Next, the buffer in the concentrate was substituted with 20 mM

15 Tris-hydrochloric acid buffer (pH 7.5) containing 0.1% Lubrol PX, to give an enzyme solution. Thirty milliliters of the resulting enzyme solution was further applied onto a porous HQ column (φ4.6 x 100 mm, manufactured by Perceptive

20 Biosystems) equilibrated with 20 mM Tris-hydrochloric acid buffer (pH 7.5) containing 0.1% Lubrol PX. Subsequently, the elution was carried out on a

concentration gradient of 0 to 0.5 M NaCl, to give an active fraction. This active fraction was applied onto hydroxyapatite column (φ7.5 x 100 mm, manufactured

25 by PENTAX) equilibrated with 1 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl and 0.1% Lubrol PX. The ceramidase did not adsorb to this column,

and was collected on an effluent column. Thereafter, the fraction was subjected

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to gel filtration chromatography by using Superose 200HR column (φ10 x 300 mm, manufactured by Amersham Pharmacia) equilibrated with 20 mM Tris-hydrochloric acid buffer (pH 7.5) containing 0.2 M NaCl and 0.3% Lubrol PX to give a purified ceramidase. As a result of the above purification procedures, 58 mg of a purified ceramidase preparation was obtained.

Various characteristics of the resulting purified ceramidase preparation were studied as described in the present specification. They were found to be as follows:

action: hydrolyzing ceramide to generate a sphingoid and fatty acid;

substrate specificity: having a substrate specificity as shown in Table 1 listed above;

optimum pH: as shown in Figure 1, the optimum pH of this ceramidase being from 7.0 to 8.0;

temperature stability: the reduced activity not being observed when treated in 20 mM Tris-hydrochloric acid buffer (pH 7.5) containing 0.1% Polidocanol (trade name: Lubrol PX) at 37°C for 24 hours, but the activity being reduced to about 30% of that before the treatment when treated for 60°C for 1 hour; and

molecular weight: about 94 kDa on SDS-PAGE (under reducing conditions); and about 73 kDa on SDS-PAGE (under reducing conditions) in this enzyme digested by glycopeptidase F.

Example 2 Partial Amino Acid Sequencing of Ceramidase

To 11 ml of a sample solution comprising 50 pmol ceramidase was added 20 mM Tris-hydrochloric acid buffer (pH 7.5) containing 0.3% Lubrol PX. The

resulting sample solution was applied onto MonoQ PCL 6/5 column (100 μ l, manufactured by Amersham Pharmacia). Subsequently, the ceramidase fraction adsorbed to the column was eluted with the same buffer containing 0.4 M NaCl. By these procedures, the ceramidase-containing fraction was concentrated to a 5 volume of 50 μ l. The resulting concentrate was subjected to SDS-polyacrylamide gel electrophoresis, and the electrophoresed gel was stained with GelCode Blue Stain reagent (manufactured by Pierce). Next, the band corresponding to the ceramidase was cut out. One-quarter of the cut-out gel fragment was subjected to extraction on 300 μ l of 0.1 M Tris-hydrochloric acid 10 buffer (pH 9.0) containing 0.1% SDS at 37°C for 16 hours. Using the resulting extract as a sample, the N-terminal amino acid sequencing of the ceramidase was performed by using G1005A Protein Sequencing System (manufactured by Hewlett-Packard) to determine an amino acid sequence N-term. SEQ ID NO: 1 of Sequence Listing shows an amino acid sequence N-term.

15 In addition, the remaining three-quarter of the cut-out gel fragment was washed with 1 ml of 0.5 M Tris-hydrochloric acid buffer (pH 9.2)/50% acetonitrile at 30°C for 45 minutes. The gel was completely dried by using nitrogen gas and centrifugal concentrator, and thereafter 10 μ l of 0.5 M Tris-hydrochloric acid buffer (pH 9.2) containing 0.5 μ g of Protease Lys-C 20 (manufactured by Wako Pure Chemical Industries, Ltd.) was added thereto. Further, 0.1 M Tris-hydrochloric acid buffer (pH 9.2) was added until the gel was completely swollen, and the mixture was kept at 37°C for 16 hours to carry 25 out the protease digestion of the ceramidase. After the termination of the reaction, the procedures of extracting with 150 μ l of 0.1% trifluoroacetic acid / 60% acetonitrile at room temperature for 1 hour were repeated twice, and

an extract was collected. This extract was subjected to reversed phase chromatography to purify the peptide fragment. The resulting peptide fragment was analyzed by Edman degradation method using the G1005A Protein Sequencing System to determine partial amino acid sequences C-46 and C-53.

5 SEQ ID NOs: 2 and 3 of Sequence Listing each shows an amino acid sequence for C-46 and C-53.

Example 3 Amplification of DNA Fragment Comprising Ceramidase Gene by PCR Method

10 Sense mix primer 53-S1 and antisense mix primer 53-A3 were designed and synthesized with a DNA synthesizer on the basis of the partial amino acid sequence C53 of the ceramidase determined in Example 2. SEQ ID NOs: 4 and 5 of Sequence Listing each show the nucleotide sequences of the primers 53-S1 and 53-A3. PCR was carried out by using these primers. PCR was carried out

15 with mouse liver cDNA plasmid library (manufactured by Takara Shuzo Co., Ltd.) as a template. PCR was carried out by a reaction of 94°C, 9 minutes; thereafter 40 cycles of reaction, wherein one cycle comprises a process consisting of 94°C, 0.5 minutes - 51°C, 0.5 minutes - 72°C, 1 minute; and further an incubation at 72°C for 7 minutes. By this PCR, a specific amplified DNA

20 fragment of a size of about 70 bp was detected on agarose electrophoresis.

25 This amplified DNA was collected from the gel, and this DNA was incorporated in pGEM-T easy vector (manufactured by Promega) to construct a recombinant plasmid. An insert DNA fragment of the plasmid was subjected to a nucleotide sequencing. As a result, a partial nucleotide sequence P-1 of this fragment was determined. SEQ ID NO: 6 of Sequence Listing shows the

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nucleotide sequence of P-1. The sequence is a sequence corresponding to the partial amino acid sequence C-53 of the ceramidase determined in Example 2. It has been confirmed that a part of the desired ceramidase gene could be obtained.

Antisense primers MA1 and MA2 were designed and synthesized on the basis of the nucleotide sequence of P-1. SEQ ID NOs: 7 and 8 of Sequence Listing each show the nucleotide sequences of the primers MA1 and MA2. In addition, sense primers T7in and T7out were designed and synthesized on the basis of the nucleotide sequence of vector pAP3neo used in the construction of the mouse liver cDNA plasmid library. SEQ ID NOs: 9 and 10 of Sequence Listing each show the nucleotide sequences of the primers T7in and T7out. Nested PCR was carried out by using these primers with the mouse liver cDNA plasmid library as a template. A 1st PCR was carried out by using the sense primer T7out and the antisense primer MA2 by a reaction at 94°C, 9 minutes; thereafter 40 cycles of reaction, wherein one cycle comprises a process consisting of 94°C, 0.5 minutes - 51°C, 0.5 minutes - 72°C, 2 minutes; and further an incubation at 72°C for 7 minutes. A 2nd PCR was carried out under the same conditions as the 1st PCR except for using the sense primer T7in and the antisense primer MA1 with a reaction mixture of the 1st PCR as a template. Consequently, an amplified DNA fragment of a size of 335 bp was obtained. This DNA fragment was used as a probe for colony hybridization described below.

Example 4 Cloning of Ceramidase Gene

A transformant resulting from introducing the mouse liver cDNA plasmid library was inoculated to a nylon filter (trade name Hybond-N⁺, manufactured by

Amersham Pharmacia) on an LB agar medium plate containing 100 μ g/ml ampicillin, and about 30000 colonies were formed per one plate of 9.5 x 13.5 cm to prepare a master filter. A replica of this filter was prepared, and the resulting replica filter was respectively treated for 5 minutes on a filter paper immersed in 5 a 10% SDS solution; 5 minutes on a filter paper immersed in a solution containing 0.5 M NaOH and 1.5 M NaCl (denaturation); 5 minutes on a filter paper immersed in 0.5 M Tris-hydrochloric acid buffer (pH 7.5) containing 3 M NaCl (neutralization); and 5 minutes on a filter paper immersed in 2 x SSC solution. Thereafter, the filter was rinsed with 2 x SSC solution. This filter was 10 air-dried, and thereafter DNA was immobilized on a filter by ultraviolet ray irradiation to be used as a filter for colony hybridization.

As the probe for the hybridization, one prepared by 32 P-labeling 0.1 μ g equivalent of the amplified DNA fragment obtained in Example 3 by using a DNA labeling kit, Ready To Go (manufactured by Pharmacia) in accordance with the 15 protocol attached to the same kit. The above-mentioned filter was placed in a hybri-bag. The pre-hybridization was carried out at 60°C for 1 hour in a hybridization solution (composition: 7% PEG6000, 10% SDS solution), and thereafter the above-mentioned labeled probe was added to the mixture so as to have a concentration of 0.006 pmol/ml, and the hybridization was carried out 20 overnight at 60°C. Next, the filter was washed three times each for 15 minutes at 60°C in the washing liquid (2 x SSC, 0.1% SDS) previously heated to 60°C. After excess water was removed from the filter, and thereafter the filter was 25 photosensitized on an imaging plate manufactured by Fuji Photo Film for 20 minutes. Thereafter, a signal was detected with BAS1000 imaging analyzer (manufactured by Fuji Photo Film). Subsequently, colonies were collected on a

master filter corresponding to a positive signal obtained by these procedures (first screening).

The collected colonies were suspended in an LB medium containing 100 μ g/ml ampicillin, and thereafter the suspension was spread over the nylon filter on an LB agar medium plate of 9.5 x 13.5 cm containing 100 μ g/ml ampicillin, and about 200 to about 1000 colonies were formed per one sheet to prepare a master filter. This filter was subjected to screening of a positive clone in the same manner as the first screening, and a third screening was further carried out by the same procedures. As a result of the third screening, the positive clone which was deduced to contain the ceramidase gene could be isolated.

A plasmid was prepared from this positive clone, and this plasmid was named plasmid pLCDase. The plasmid was digested with various kinds of restriction enzymes or a combination of plural restriction enzymes. Thereafter, each of the formed DNA fragments was subcloned, and its nucleotide sequence was analyzed. By the above procedures, an entire nucleotide sequence of the DNA fragment inserted in the plasmid pLCDase was determined. The sequence is shown in SEQ ID NO: 11 of Sequence Listing. In addition, the nucleotide sequence of the open reading frame (ORF) found in the sequence and the amino acid sequence of the polypeptide encoded thereby are each shown in SEQ ID NOs: 12 and 13 of Sequence Listing. Further, the restriction enzyme map of the above-mentioned DNA fragment and the position of the open reading frame contained in the DNA fragment are shown in Figure 2.

As a result of analysis of the nucleotide sequence of the above-mentioned ORF, there were confirmed that the nucleotide sequence comprises a nucleotide

sequence encoding a partial amino acid sequence of the ceramidase elucidated by Example 2, and that this ORF encodes ceramidase. In addition, when the amino acid sequence of the ceramidase encoding the ORF was compared with the amino acid sequence of the ceramidase shown in SEQ ID NO: 1 of Sequence Listing, it was shown that the ceramidase purified from the mouse liver lacked the peptide of the N-terminal portion of the polypeptides encoded by the above-mentioned ORF. In other words, it was shown that the ceramidase was converted to a mature enzyme by going through the processing in which its N-terminal portion was removed after the translation. SEQ ID NO: 14 of Sequence Listing shows an amino acid sequence of the mature ceramidase, and SEQ ID NO: 15 of Sequence Listing shows a nucleotide sequence of the mature ceramidase, respectively.

Example 5 Expression of Ceramidase Gene

To CHO cells cultured in a 10% FCS-containing α -MEM medium in a dish having a diameter of 35 mm (3×10^5 cells/dish) were added 1 μ g of the plasmid pLCDase obtained in Example 4 and 5 μ l of lipofectamine (manufactured by Life Technologies), thereby transducing a ceramidase gene into CHO cells. The cells were cultured at 37°C for 24 hours, and thereafter suspended in 100 μ l of 10 mM Tris-hydrochloric acid (pH 7.5) containing 0.1% Triton X-100, and the cells were disrupted. The determination of the activity on the ceramidase of which substrate was the above-mentioned C12-NBD-ceramide was made for the resulting solution containing disrupted cells. As a result, it was confirmed that the ceramidase in the cells intensively expressed about 1000 times more than that of the control cells in which pLCDase was not

transduced.

Further, the amount of ceramide in the cells was determined in accordance with the method described in *Analytical Biochemistry*, 244, 291-300 (1997). As a result, it was confirmed that those pLCDase-transduced cells had a significantly reduced amount of ceramide, as compared to the control cells into which pLCDase was not transduced.

Example 6 Cloning of Ceramidase Gene from Mouse Brain

A nitrocellulose membrane (Schleicher & Schuell, PROTRAN BA85 10 0.45 mm being used with a diameter of 82 mm) was placed on an LB agar medium plate containing 100 μ g/ml ampicillin, and the mouse brain cDNA library (LIFE TECHNOLOGIES, SUPERSOFT Mouse Brain cDNA Library) was spread on each of 10 plates, so as to be about 200000 colonies per one plate, and cultured at 37°C for 10 hours. *E. coli* grown on the nitrocellulose membrane 15 was transferred to a nylon membrane [PALL Gelman Laboratory, biodyne A diameter 82 mm (1.2 mm)], and each nylon membrane was placed on an ampicillin plate, and thereafter cultured at 37°C for 3 hours. The nitrocellulose membrane was stored at 4°C as a master filter, and the nylon membrane was placed on chloramphenicol plate, and cultured at 37°C for 16 hours. Colonies 20 were transferred to a fresh nylon membrane from the nylon membrane, and front and back sides of the membrane were each treated for 5 minutes with 1 ml of a denaturation solution (0.5 M NaOH/1.5 M NaCl) in a state where each pair of nylon membranes was overlaid. Similarly, the nylon membranes were treated 25 with 1 ml of a neutralization solution [0.5 M Tris-HCl (pH 7.4) / 1.5 M NaCl] for 5 minutes. The nylon membrane was peeled off, and the treated membrane was

air-dried, and thereafter baked at 80°C for 2 hours. Thereafter, the baked membrane was shaken with 200 ml of a pre-rinsing liquid [5 x SSC / 0.5% SDS / 1 ml EDTA (pH 8.0)], and disrupted *E. coli* residues were wiped off, and washed with 2 x SCC. The hybridization was carried out with 40 ml of a hybridization solution [0.5 M Church phosphate buffer/ 7% SDS / 1 mM EDTA] at 65°C for 2 hours, and thereafter the hybridization was carried out at 65°C for 16 hours in 40 ml of the hybridization solution containing the denatured probe. As the probe, an EcoRI-EcoRI fragment of a size of 2.7 kbp of the plasmid pAPLCD carrying mouse ceramidase gene was used. After the termination of the hybridization, washing with 100 ml of a washing liquid (40 mM Church phosphate buffer/ 1% SDS) at 65°C for 15 minutes was carried out twice. Further, washing with 100 ml of a high stringent washing liquid (0.2 x SSC / 0.1% SDS) at 65°C for 15 minutes was carried out. The membrane was air-dried, and thereafter exposed on an IP-plate for 1 hour, and analyzed with BAS 1500. The positive part of the nitrocellulose membrane was cut out in a diameter of about 6 mm, and suspended in 1 ml of the LB medium. Thereafter, a 200 ml sample prepared by diluting the positive part 4000-folds was spread over an ampicillin-containing LB plate, and a 2nd screening was carried out.

Similarly, a 200 ml sample prepared by diluting the positive part 10000-folds was spread over an ampicillin-containing LB plate, to prepare a library. A 3rd screening was carried out by using this library. The isolated clone (pSBCD) was subcloned, and thereafter the nucleotide sequence was determined by a conventional method. This sequence is shown in SEQ ID NO: 16.

In the above-mentioned sequence, an ORF having the identical sequence as the ceramidase gene derived from mouse liver described in Example 4 was

found. Incidentally, the sequences of 5' non-translational region and 3' non-translational region were different from those derived from the mouse liver. In addition, the isolated plasmid pSBCD was transduced into CHO cells in the same manner as in Example 5. As a result, it was found that the ceramidase was expressed in the cells.

5

Example 7 Genomic Cloning of Human Ceramidase Gene

Sense primer U1107 having the sequence of SEQ ID NO: 17 of Sequence Listing and antisense primer L1311 having the sequence of SEQ ID NO: 18 of Sequence Listing were synthesized on the basis of the sequence of the ceramidase gene derived from mouse liver determined in Example 4. The sequence of U1107 primer corresponds to a sequence consisting of base nos.: 1107 - 1130 of SEQ ID NO: 12 of Sequence Listing, and the sequence of L1311 primer corresponds to a nucleotide sequence complementary to a sequence consisting of base nos.: 1311 - 1334 of SEQ ID NO: 12 of Sequence Listing.

10 Genomic DNA was purified from human hepatoma cell Huh7 by a conventional method. PCR was carried out by using the U1107 primer and the L1311 primer with 625 ng of the resulting genomic DNA as a template. PCR was carried out by a reaction at 94°C, 9 minutes; thereafter 40 cycles of reaction, 15 wherein one cycle comprises a process consisting of 94°C, 0.5 minutes - 55°C, 0.5 minutes - 72°C, 3 minutes; and further an incubation at 72°C for 7 minutes. Thereafter, the resulting reaction product was subjected to agarose 20 electrophoresis. As a result, it was confirmed that a DNA fragment of a size of 25 about 2 kbp was amplified by the PCR.

This DNA fragment was collected from the gel by using Sephaglas (manufactured by Pharmacia), and the resulting fragment was incorporated in pGEM-T easy vector (manufactured by Promega) to construct a recombinant plasmid. Next, the nucleotide sequence of the DNA fragment insert of the resulting plasmid was determined. The above-mentioned sequence was a sequence corresponding to 96289 - 98478 of the Accession No. AC012131 Complement registered in the GenBank Data Base. In addition, the amino acid sequence encoded by the above-mentioned sequence was analyzed. As a result, a region encoding an amino acid sequence showing homology to a region of the amino acid nos.: 370 - 444 of the amino acid sequence of the ceramidase derived from mouse liver of SEQ ID NO: 4 of Sequence Listing and a region encoding an amino acid sequence showing homology were found.

Incidentally, AC012131 also has homology with the ceramidase gene derived from mouse liver determined in Example 4.

15

SEQUENCE LISTING FREE TEXT

In the amino acid sequence of SEQ ID NO: 1, each of Xaa in the amino acid numbers: 7, 9 and 13 stands for an unknown amino acid.

SEQ ID NO: 4 is a sequence of synthetic oligonucleotide primers. In the above-mentioned sequence, each of n in the base numbers: 6, 9 and 15 stands for G, A, T or C.

SEQ ID NO: 5 is a sequence of synthetic oligonucleotide primers. In the above-mentioned sequence, each of n in the base numbers: 3, 6 and 15 stands for G, A, T or C.

SEQ ID NO: 7 is a sequence of synthetic oligonucleotide primers.

SEQ ID NO: 8 is a sequence of synthetic oligonucleotide primers.

SEQ ID NO: 9 is a sequence of synthetic oligonucleotide primers.

SEQ ID NO: 10 is a sequence of synthetic oligonucleotide primers.

SEQ ID NO: 11 is a sequence of synthetic oligonucleotide primers.

5 SEQ ID NO: 17 is a sequence of synthetic oligonucleotide primers.

SEQ ID NO: 18 is a sequence of synthetic oligonucleotide primers.

INDUSTRIAL APPLICABILITY

10 According to the present invention, a gene encoding a neutral/alkaline ceramidase derived from a mammal is provided, and a genetic engineering method for producing a ceramidase using the gene. Also, the oligonucleotide probe and primer of the present invention are useful for detection of the above-mentioned gene, and can be applied to studies on *in vivo* ceramide metabolism.

15 Further, according to the present invention, an antisense nucleic acid (DNA, RNA) of the gene of the present invention is provided. The gene and its antisense nucleic acid are useful in the control of the ceramidase activity and in the regulations of the *in vivo* ceramide metabolism system. Therefore, a method of regulating an amount of ceramide which can be applied to treatments of diseases caused by the abnormal amount of ceramide is provided.

CLAIMS

1. A gene having a nucleotide sequence of a nucleic acid selected from the group consisting of:
- 5 (A) a nucleic acid encoding a polypeptide having the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing or a partial sequence thereof, the polypeptide possessing a ceramidase activity;
- 10 (B) a nucleic acid having a nucleotide sequence of SEQ ID NO: 15 of the Sequence Listing or a partial sequence thereof and encoding a polypeptide possessing a ceramidase activity;
- 15 (C) anucleic acid encoding a polypeptide consisting of an amino acid sequence resulting from deletion, addition, insertion or substitution of at least one amino acid residue in the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing, the polypeptide possessing a ceramidase activity;
- 20 (D) a nucleic acid consisting of a nucleotide sequence resulting from deletion, addition, insertion or substitution of at least one base in the nucleotide sequence of SEQ ID NO: 15 of the Sequence Listing and encoding a polypeptide possessing a ceramidase activity;
- (E) a nucleic acid capable of hybridizing to a complementary strand of a nucleic acid of any one of the above (A) to (D), under stringent conditions, and encoding a polypeptide possessing a ceramidase activity; and
- 25 (F) a nucleic acid having a nucleotide sequence different from the nucleic acid of any one of above (A) to (E) via degeneracy and encoding a polypeptide possessing a ceramidase activity.

2. The gene according to claim 1, wherein the ceramidase activity of the polypeptide is capable of being detected by the following steps:
- (a) incubating a gene expression product in a reaction mixture [composition: 5 550 pmol of C12-NBD-ceramide and 1.0% (W/V) sodium cholate in 20 μ l of 25 mM Tris-hydrochloric acid buffer (pH 7.5)] at 37°C for 30 minutes, to react the mixture; and
- (b) detecting generation of a C12-NBD-fatty acid in the reaction product.
3. The gene according to claim 1 or 2, wherein the polypeptide exhibits at 10 least the following characteristics:
- (i) action: hydrolyzing ceramide, to generate sphingoid and a fatty acid;
- (ii) substrate specificity: hydrolyzing N-acylsphingosine; but not acting on galactosylceramide, sulfatide, GM1a, and sphingomyelin;
- (iii) optimum pH: being from 7.0 to 8.0; and
- 15 (iv) lowering of the activity not being found when treated in 20 mM Tris-hydrochloric acid (pH 7.5) containing 0.1% polidocanol at 37°C for 24 hours, but the activity lowering by a treatment at 60°C for 1 hour to about 30% of the activity before the treatment.
- 20 4. A recombinant DNA comprising the gene of any one of claims 1 to 3.
5. An expression vector for a microorganism, an animal cell or a plant cell, comprising the gene of any one of claims 1 to 3 or the recombinant DNA of claim 4.

6. A transformant carrying the expression vector of claim 5.
7. A method for producing a polypeptide possessing a ceramidase activity, characterized by culturing the transformant of claim 6 under conditions appropriate for expression of the ceramidase gene and production of the polypeptide encoded by the gene, and collecting a polypeptide possessing a ceramidase activity from the resulting culture.
8. A polypeptide having the amino acid sequence of SEQ ID NO: 14 of the Sequence Listing or a partial sequence thereof and possessing a ceramidase activity.
9. A polypeptide possessing a ceramidase activity, encoded by the gene of any one of claims 1 to 3.
10. The polypeptide according to claim 8 or 9, wherein the ceramidase activity is capable of being detected by the following steps:
- (a) incubating a gene expression product in a reaction mixture [composition: 550 pmol of C12-NBD-ceramide and 1.0% (W/V) sodium cholate in 20 μ l of 25 mM Tris-hydrochloric acid buffer (pH 7.5)] at 37°C for 30 minutes, to react the mixture; and
- (b) detecting generation of a C12-NBD-fatty acid in the reaction product.
11. An antisense DNA which is complementary to the gene of any one of claims 1 to 3 or a part thereof.

12. An antisense RNA which is complementary to the gene of any one of claims 1 to 3 or a part thereof.
- 5 13. An expression vector comprising the antisense DNA of claim 11.
14. An oligonucleotide probe or primer, capable of specifically hybridizing to the gene of any one of claims 1 to 3 or a complementary strand thereof.
- 10 15. An antibody or a fragment thereof, capable of specifically binding to the polypeptide of any one of claims 8 to 10.
- 15 16. A method for detecting a gene encoding a polypeptide possessing a ceramidase activity, comprising using the oligonucleotide probe or primer of claim 14.
- 20 17. A kit for the use in detection of a gene encoding a polypeptide possessing a ceramidase activity, comprising the oligonucleotide probe and/or primer of claim 14.
18. A method for detecting a polypeptide possessing a ceramidase activity, comprising using the antibody or a fragment thereof of claim 15.
- 25 19. A kit for the use in detection of a polypeptide possessing a ceramidase activity, comprising the antibody or a fragment thereof of claim 15.

20. A method of controlling an amount of ceramide in a cell and/or in a tissue, characterized by introducing the gene of any one of claims 1 to 3 or an antisense nucleic acid thereof into the cell and/or the tissue, thereby controlling the amount of ceramide in the cell and/or in the tissue.

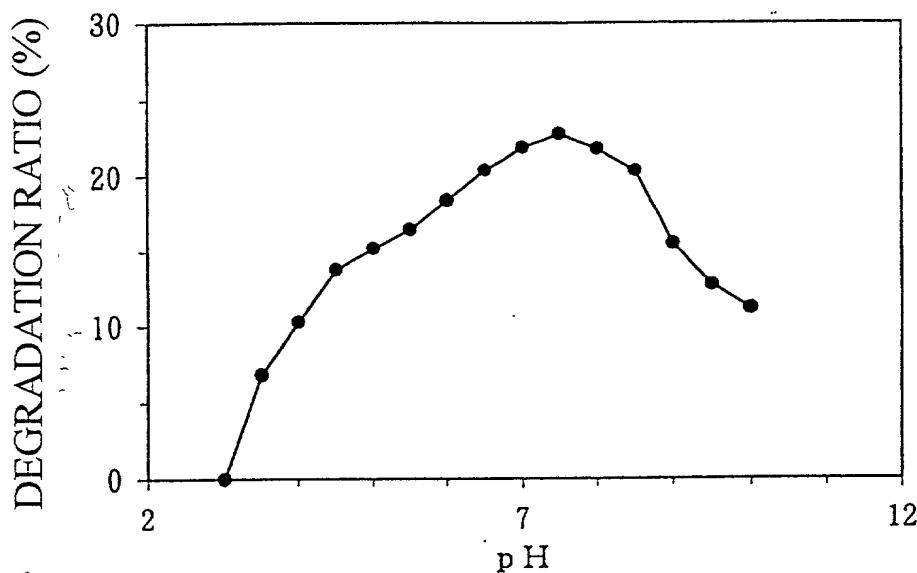
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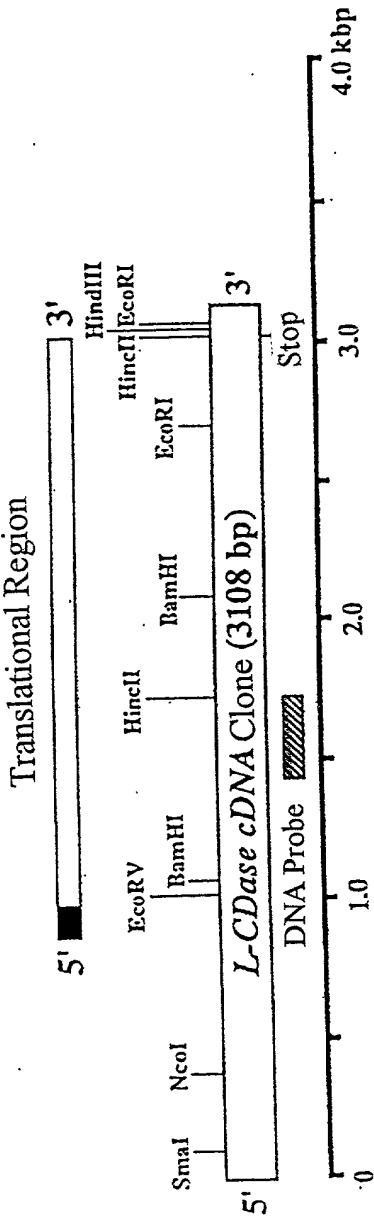
FIG. 1



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FIG. 2



I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

1422-0493P

17

Raymond C. Stewart	(Reg. No. <u>21,066</u>)	Terrell C. Birch	(Reg. No. <u>19,382</u>)
Joseph A. Kolasch	(Reg. No. <u>22,463</u>)	James M. Slattery	(Reg. No. <u>28,380</u>)
Bernard L. Sweeney	(Reg. No. <u>24,448</u>)	Michael K. Mutter	(Reg. No. <u>29,680</u>)
Charles Gorenstein	(Reg. No. <u>29,271</u>)	Gerald M. Murphy, Jr.	(Reg. No. <u>28,977</u>)
Leonard R. Svensson	(Reg. No. <u>30,330</u>)	Terry L. Clark	(Reg. No. <u>32,644</u>)
Andrew D. Meikle	(Reg. No. <u>32,868</u>)	Marc S. Weiner	(Reg. No. <u>32,181</u>)
Joe McKinney Muncy	(Reg. No. <u>32,334</u>)	C. Joseph Faraci	(Reg. No. <u>32,350</u>)
Donald J. Daley	(Reg. No. <u>34,313</u>)	John W. Bailey	(Reg. No. <u>32,881</u>)
John A. Castellano	(Reg. No. <u>35,094</u>)		

Send Correspondence to: **BIRCH, STEWART, KOLASCH & BIRCH, LLP**

P.O. Box 747 • Falls Church, Virginia 22040-0747

Telephone: (703) 205-8000 • Facsimile: (703) 205-8050

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or
Sole Inventor
Insert Name of
Inventor
Insert Date This
Document is Signed

Insert Residence
Insert Citizenship

Insert Post Office
Address
Full Name of Second
Inventor, if any.
see above

Full Name of Third
Inventor, if any
see above

Full Name of Fourth
Inventor, if any
see above

Full Name of Fifth
Inventor, if any
see above

GIVEN NAME <u>Makoto</u>		FAMILY NAME <u>ITO</u>	INVENTOR'S SIGNATURE <u>Makoto Ito</u>	DATE* <u>Aug. 28, 01</u>
Residence (City, State & Country) <u>Fukuoka-shi, Fukuoka 819-0013 Japan</u>		CITIZENSHIP <u>Japan</u>		
POST OFFICE ADDRESS (Complete Street Address including City, State & Country) <u>34-1, Atagohama 4-chome, Nishi-ku, Fukuoka-shi, Fukuoka 819-0013 Japan</u>				
GIVEN NAME		FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Residence (City, State & Country)		CITIZENSHIP		
POST OFFICE ADDRESS (Complete Street Address including City, State & Country)				
GIVEN NAME		FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
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POST OFFICE ADDRESS (Complete Street Address including City, State & Country)				

* DATE OF SIGNATURE

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10/21

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 245 250 255
 Ser Pro Asn Ile Leu Gly Pro His Cys Val Asn Thr Gly Glu Ser
 260 265 270
 Cys Asp Asn Asp Lys Ser Thr Cys Pro Asn Gly Gly Pro Ser Met
 275 280 285
 Cys Met Ala Ser Gly Pro Gly Gln Asp Met Phe Glu Ser Thr His
 290 295 300
 Ile Ile Gly Arg Ile Ile Tyr Gln Lys Ala Lys Glu Leu Tyr Ala
 305 310 315
 Ser Ala Ser Gln Glu Val Thr Gly Pro Val Leu Ala Ala His Gln
 320 325 330
 Trp Val Asn Met Thr Asp Val Ser Val Gln Leu Asn Ala Thr His
 335 340 345
 Thr Val Lys Thr Cys Lys Pro Ala Leu Gly Tyr Ser Phe Ala Ala

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Phe Arg Glu Ala Ile Lys Lys Glu Phe Ala Leu Tyr Gly Met Lys
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485 490 495
Ala Ser Thr Ile Tyr Gly Pro His Thr Leu Ser Ala Tyr Ile Gln
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Leu Phe Arg Asp Leu Ala Lys Ala Ile Ala Thr Asp Thr Val Ala
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530 535 540
Ala Ser Leu Ile Pro Asn Ile Ala Asp Arg Ala Pro Ile Gly Lys
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His Phe Gly Asp Val Leu Gln Pro Ala Lys Pro Glu Tyr Arg Val
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